

# **EXHIBIT A**

# Death of HT29 adenocarcinoma cells induced by TNF family receptor activation is caspase-independent and displays features of both apoptosis and necrosis

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## Abstract

The HT29 adenocarcinoma is a common model of epithelial cell differentiation and colorectal cancer and its death is an oft-analyzed response to TNF family receptor signaling. The death event itself remains poorly characterized and here we have examined the involvement of caspases using pan-caspase inhibitors. zVAD-fmk did not block death of HT29 cells in response to activation of the Fas, TRAIL, TNF, TWEAK and LT $\beta$  receptors. The secondary induction of TNF or the other known *bona fide* death inducing ligands did not account for death following LT $\beta$  receptor activation indicating that TNF family receptors can trigger a caspase-independent death pathway regardless of the presence of canonical death domains in the receptor. To provide a frame of reference, the phenotype of HT29 death was compared to four other TNF family receptor triggered death events; Fas induced Jurkat cell apoptosis, TNF/zVAD induced L929 fibroblast necrosis, TNF induced death of WEHI 164 fibroblastoid cells and TNF/zVAD induced U937 death. The death of HT29 and U937 cells under these conditions is an intermediate form with both necrotic and apoptotic features. The efficient coupling of TNF receptors to a caspase-independent death event in an epithelial cell suggests an alternative approach to cancer therapy.

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**Keywords:** TNF; caspase-independent; apoptosis; necrosis

**Abbreviations:** AIF, apoptosis inducing factor; DAPI, 4,6 diamino-phenyl-2-phenylindole; IFN $\gamma$ , interferon- $\gamma$ ; JNK, cJun N terminal kinase; LT $\beta$ R, lymphotoxin- $\beta$  receptor; MTT, 3-(4,5-dimethylthiazol-2-yl) 2,5 diphenyltetrazolium bromide; PCD, programmed cell death; PI, propidium iodide; ROS reactive oxygen species; TNFR, tumor necrosis factor receptor; TRAF, Tumor necrosis factor receptor associated factors; TRAIL-R, TRAIL receptor; 7AAD, 7-amino actinomycin D

## Introduction

Receptors in the TNF family can initiate both canonical apoptotic and necrotic death events.<sup>1</sup> Prototypical apoptosis follows activation of the Fas receptor on T cells leading to caspase activation and a cascade of events eventually culminating in the various hallmarks of apoptosis.<sup>2,3</sup> Yet even in this familiar case, Fas/FADD can trigger necrosis in T cells in the absence of caspase signaling.<sup>4,5</sup> In the well-studied L929 fibroblast line, Fas activation triggers apoptosis while TNF initiates a necrotic event that is actually enhanced by caspase inhibition.<sup>1</sup> Similarly, TNF signaling in the presence of caspase inhibitors was reported to lead to the necrosis of NIH3T3 fibroblasts and the myeloid U937 cell line.<sup>6</sup> It has been dogmatic that apoptosis is only initiated by those TNF family receptors containing an intracellular death domain capable of coupling to the caspase machinery. Paradoxically, however, several TNF family receptors lacking canonical death domains can trigger an apoptotic-like death. This phenomenon results from the secondary induction of TNF secretion in some cell lines leading to classical apoptosis via the death domain containing p55 TNF receptor.<sup>7,8</sup> Therefore the question of whether a non-death domain containing receptor in the TNF family can directly trigger a death pathway remains unresolved.

Two additional categories of death, apoptosis-like programmed cell death (PCD) and necrosis-like PCD, were defined as intermediates between canonical apoptosis and necrosis.<sup>9</sup> Cells undergoing both intermediate types of death display some aspects of apoptotic mechanisms, but are distinguished by the patterns of chromatin condensation. These intermediates may correlate with several of the variations of PCD observed *in vivo*.<sup>10,11</sup> The heterogeneity of death mechanisms has been underscored by recent observations questioning the exact role played by caspases in some critical physiological events. For example, lymphocytes can undergo caspase-independent death in response to a number of stimuli.<sup>12</sup> Surprisingly, caspase activity is actually required for T-cell proliferation.<sup>13</sup> Genetic deletion of caspases has revealed some critical developmental roles, but in general the effects have been underwhelming, leading to speculation of alternate physiologically relevant PCD mechanisms. Release of apoptosis inducing factor (AIF) from the mitochondria leads to caspase-independent PCD and represents a potential mechanism to account for some of these observations.<sup>14</sup> From these various studies, it appears that collapse of the mitochondrial membrane potential, cytochrome *c* and AIF release, zeiosis, phosphatidylserine translocation, chromatin cleavage to 50 kDa pieces, condensation and margination can be triggered independently of caspase activity. DNA cleavage into

nucleosome sized pieces appears dependent upon caspase activation of the CAD nuclease.<sup>9</sup>

The adenocarcinoma cell line HT29 is often used in the TNF field as an assay system and this cell also serves as a model of intestinal epithelial cell differentiation and colorectal cancer. We are interested in the death of HT29 cells that follows activation of various members of the TNF receptor family and in particular the death induced by an anti-LT $\beta$ R agonist antibody.<sup>15–21</sup> This antibody has demonstrated anti-tumor potential *in vivo*.<sup>15</sup> Death of this cell when induced by TNF family receptors is somewhat unusual as it is relatively slow and when analyzed with cells grown on plastic, interferon- $\gamma$  (IFN- $\gamma$ ) co-signaling is required for death.<sup>22,23</sup> Moreover, HT29 death is enhanced by IFN- $\gamma$  even when initiated by non-TNF related proapoptotic stimuli such as staurosporine, cisplatin and ceramides, but not by agents that induce necrosis.<sup>19</sup> Thus IFN- $\gamma$  affects downstream signaling events and may toggle the cell's response to receptor activation between death and differentiation.

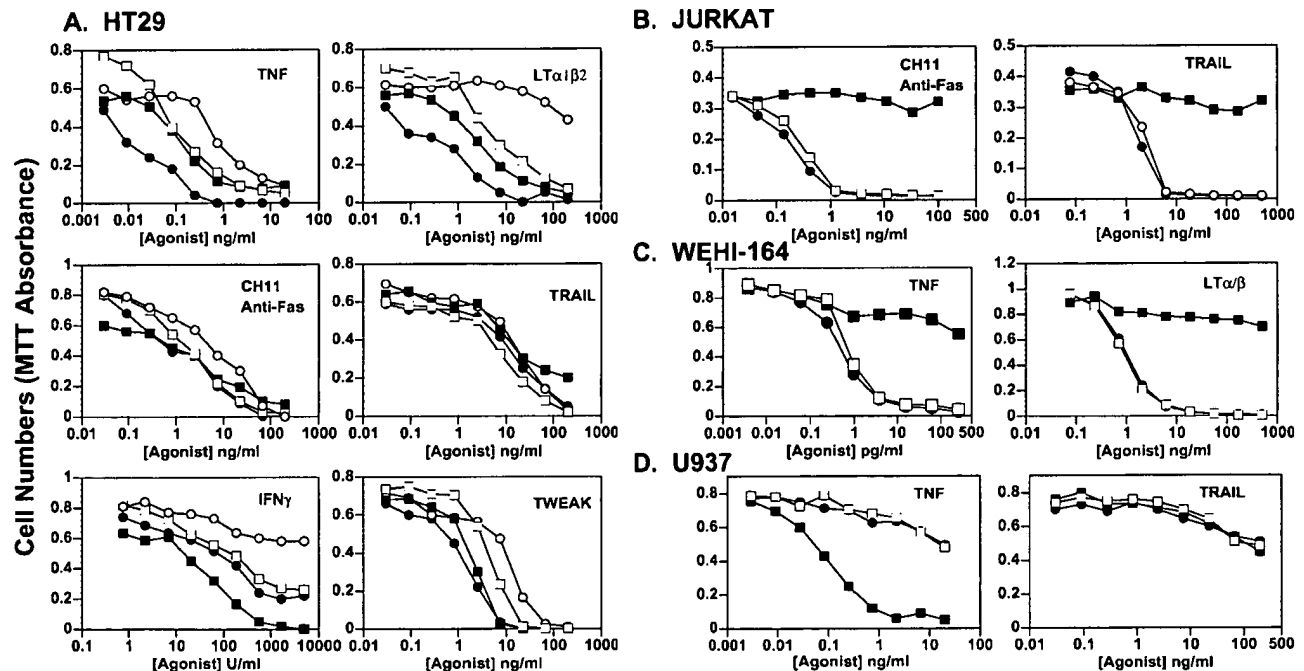
Given this background, we were interested in characterizing the death initiated by LT $\beta$ R and TNFR activation in the human HT29 and murine WEHI 164 cell systems.<sup>15,24</sup> To provide a frame-of-reference, HT29 cells were compared to well-characterized apoptotic and necrotic events in Jurkat and L929 cells and the TNF/zVAD-fmk induced death of U937 cells. All death events initiated by receptors in the

TNF family have been reported to be caspase-dependent except TNF/zVAD induced necrosis in L929, NIH3T3 and U937 cells, LIGHT triggered Hep3B death and recent reports on signaling via the Taj and Wengen receptors.<sup>25,26</sup> Therefore, we have questioned whether death of the epithelial HT29 cell induced by TNF family receptors either with or without death domains is actually caspase-dependent and more specifically whether this death is directly dependent on the secondary secretion of other TNF family ligands, e.g. TNF, Fas-L, TWEAK or TRAIL. Here we find that caspase-independent death of HT29 cells can be induced by activation of TNF family receptors either with or without death domains and the mechanism does not involve the secondary generation of death-inducing ligands.

## Results

### HT29 death is not blocked by the general caspase inhibitor zVAD-fmk

We used the general caspase inhibitor zVAD-fmk to assess the role of caspases in HT29 death in a 3–4 day growth assay (Figure 1A). In this well-defined assay, the lack of growth is due to death and not simply anti-proliferative effects.<sup>15,18–20</sup> Death induced by activation of TNF family receptors including TNF-R, LT $\beta$ R, Fas, TRAIL-R, IFN- $\gamma$ -R and TWEAK-R was not blocked by zVAD-fmk treatment (Figure 1A, compare open



**Figure 1** Effects of the general caspase inhibitor zVAD-fmk on cell viability in 3–4 day growth assays using an MTT readout. Cell death was triggered by activation of the TNF family of receptors in various cell types. Cells were treated with the indicated concentrations of the cytokines TNF, TRAIL, TWEAK, LT $\alpha$ 1/ $\beta$ 2 and/or the anti-Fas agonist mAb in the presence of no additions (closed circle), 50  $\mu$ M zVAD-fmk, final solvent concentration 0.25% DMSO (closed square), 0.25% DMSO alone (open square) or 1% DMSO alone (open circle). Shown are the effects on (A) a human adenocarcinoma line HT29 (block of six graphs), (B) a human T cell line Jurkat, (C) a mouse fibroblastoid line WEHI 164 and (D) a human monocytic leukemia line U937. Human ligands were used in each case except for murine LT $\alpha$ 1/ $\beta$  with WEHI 164 cells.<sup>24</sup> Wells with TRAIL also contained anti-FLAG mAb M2 (1  $\mu$ g/ml) to further crosslink the TRAIL-R. IFN $\gamma$  (80 U/ml) was present only in the HT29 experiments with TNF ligands. In the HT29 panel where IFN- $\gamma$  alone was used, the x-axis is plotted as units of antiviral activity per ml. Direct visual examination confirmed that the decreased MTT readout was due to cell death and not inhibition of cell proliferation in each assay. Data represents the average of duplicate wells and all MTT growth experiments were carried out at least three times with similar results

and closed squares). zDEVD-fmk (100  $\mu$ M), zVAD-fmk plus zDEVD-fmk, zAAD-cmk (100  $\mu$ M), Ac-YVAD-cmk (50  $\mu$ M) and Boc-D-fmk (BD-fmk, another general caspase inhibitor,<sup>27</sup> 10–100  $\mu$ M) similarly had no effect on HT29 death (data not shown). Pretreatment of the HT29 cells for 30 min with zVAD-fmk or addition of fresh zVAD-fmk after 2 days did not alter the results. Curiously, LT $\beta$ R or IFN- $\gamma$  triggered HT29 death was inhibited by higher concentrations of dimethylsulfoxide (Figure 1A, open circles). This effect was maximal at 1% (data not shown) and indicates a need for caution in the use of this solvent with some cell types. This effect may be due to induction of cellular differentiation by this solvent and the apparent selectivity for LT $\beta$ R may result from the relatively slow kinetics of this death event. HT29 cells are known to exist in multiple states indicating a proclivity for differentiation events.<sup>32,33</sup> In support of this hypothesis, the differentiating agents, sodium butyrate, 1,25 dihydroxyvitamin D3 and dimethylformamide were protective while retinoic acid and dexamethasone did not have an effect (data not shown).

For comparison, several other well-studied death events were examined. The death of the Jurkat T cell lymphoid line following Fas or TRAIL-R activation (Figure 1B) and the SKW 6.4 B cell line following Fas activation (data not shown) was completely blocked by zVAD-fmk. Importantly, these data demonstrate that the caspase inhibitor remained active throughout these long term assays. Similarly, zVAD-fmk completely blocked the death induced by TNF-R or LT $\beta$ R activation in the WEHI 164 fibroblastoid cell line (Figure 1C). It is interesting that WEHI-164 death following LT $\beta$ R activation was caspase-dependent since this receptor lacks a canonical death domain. The U937 line also dies in response to TNF signaling (Figure 1D), although the ATCC derived isolate used in these experiments required high concentrations of TNF in agreement with the previously noted poor sensitivity.<sup>30</sup> It was reported and we had independently made the same observation that TNF-induced U937 death was dramatically augmented by zVAD-fmk (Figure 1D) and BD-fmk (data not shown).<sup>6</sup> TRAIL induced death, however, was not enhanced by zVAD-fmk (Figure 1D). LT $\beta$ R activation had no effect on the U937 cells with or without zVAD-fmk (data not shown). The TNF-induced death of THP-1 cells, another human monocytic line, was also potentiated by zVAD-fmk albeit less dramatically, while HL-60 cells were not affected (data not shown). A similar enhancement of TNF induced death by zVAD-fmk was reported with the mouse fibroblastoid lines L929 and NIH3T3.<sup>6,31</sup> Inhibition of caspase activity by zVAD-fmk in these systems was directly verified using fluorogenic YVAD, DEVD and IETD, as representative substrates for group I, group II and group III caspases.<sup>32</sup> Even at times up to 48 h after addition of the agonist, caspase activity was not detected in zVAD-fmk treated HT29 cells and similar results were observed for the other lines at 3 h (data not shown).

### Induction of endogenous TNF production does not mediate LT $\beta$ R-induced death in HT29 or WEHI 164 cells

In some cells, signaling through TNF family receptors lacking death domains, e.g. TNFRII, CD40, CD30 and the TWEAK

receptor involved upregulation of endogenous TNF synthesis thereby inducing death indirectly.<sup>7,8</sup> Accordingly in these systems, blocking antibodies to either TNF or TNFR1 inhibit the death induced by ligands that bind to nondeath-domain containing receptors. To determine whether the death of HT29 cells or WEHI 164 cells by LT $\beta$ R signaling occurred by upregulation of TNF synthesis, we used an immobilized agonistic anti-human LT $\beta$ R mAb and a murine LT $\alpha/\beta$  ligand to activate the human and murine receptors.<sup>15,24</sup> Table 1 shows that blockade of TNF, FasL, TRAIL or TWEAK with either neutralizing antibodies or soluble receptors did not affect the LT $\beta$ R triggered death of either cell type. Only LT $\alpha/\beta$  inhibitors were able to block the death event in this setting. It can be seen in this table that all of the inhibitors used in this study were capable of inhibiting their respective targets in positive control systems even in these long term assay formats. Therefore, secondary induction of the expression of other death inducers does not appear to account for the effects of LT $\beta$ R signaling.

### Nuclear changes and phosphatidylserine translocation in these death events

Historically, TNF/IFN $\gamma$  triggered HT29 death was considered to be apoptotic since the nuclei condense, annexin V binding increases and clear cytoplasmic blebs devoid of organelles can be observed.<sup>15,18–20,33–35</sup> However, not all facets of this death are consistent with an apoptotic process. The cytoplasmic blebs are similar to those described on L929 cells undergoing Fas-induced apoptosis; however, these blebs may be more indicative of necrosis or post-apoptotic necrosis.<sup>1</sup> Likewise, in some studies, DNA cleavage in dying HT29 cells was limited to the generation of the large 50–200 kb fragments often seen in dying epithelial cells rather than the complete nucleosome laddering viewed as a hallmark of conventional apoptosis.<sup>34–36</sup> To determine if the cells undergo the chromatin condensation and margination characteristic of apoptosis, an electron microscopic analysis was carried out. Following TNFR, Fas or TWEAK-R activation, the chromatin condensed and localized to the nuclear membrane, i.e. margined. An example of an anti-Fas treated dying HT29 cell is shown in Figure 2. At this time point, the bulk of the IFN- $\gamma$  treated cells were normal in appearance (untreated control not shown) while dying TNF and TWEAK treated HT29 cells resembled anti-Fas treated cells. The dying cells exhibited rounding and slight cellular swelling which may reflect necrotic or post-apoptotic necrotic events, yet the loss of microvilli, disruption of the mitochondrial cristae without apparent mitochondrial swelling and dilation of the endoplasmic reticulum are features of apoptosis.

Some DNA fragmentation can be detected in HT29 cells following activation of the TNF receptors or Fas.<sup>15,18,37,38</sup> To determine the level of DNA fragmentation in these experiments, a FACS analysis of PI stained cells was undertaken to monitor DNA cleavage. TNF or anti-LT $\beta$ R agonist antibody treated HT29 cells contained subdiploid DNA and its generation was blocked by zVAD-fmk treatment (Table 2). Likewise, anti-Fas-treated Jurkat cells and TNF-treated U937 cells exhibited increased levels of

**Table 1** The lack of effects of inhibitors of various death inducing TNF ligands on the LT $\beta$ R activation triggered death of HT29 and WEHI 164 cells

Cell	Activating agent	Agonist concentration at the ED50 (ng/ml) <sup>a</sup>					
		None	Anti-hFasL	Anti-hTNF	Anti-TWEAK	hTRAIL-R2-Ig	hLTBR-Ig
HT29	Anti-LT $\beta$ R	1.1	2.2	1.4	1.4	1.7	>300
	hTNF	0.1	—	>20	—	—	—
	hTRAIL	0.2	—	—	—	70	—
	hFasL	3	>5000	—	—	—	—
	hTWEAK	10	—	—	>500	—	—
WEHI-164		None	Anti-mFasL	Anti-mTNF	Anti-TWEAK	mTRAIL-R2-Ig	mLT $\beta$ R-Ig
	mLT $\alpha/\beta$	1.0	0.9	1.0	1.2	1.0	>100
	mTNF	0.0001	—	>10	—	—	—
	mTRAIL	300	—	—	—	>15000	—
	mTWEAK	0.1	—	—	>200	—	—
JURKAT	mFasL	400	>40 000	—	—	—	—

<sup>a</sup>MTT proliferation assays were carried out in the absence or presence of the inhibitors of the appropriate mouse or human forms as defined in Materials and Methods. An agonistic anti-LT $\beta$ R mAb, CBE11, was used to activate this receptor on HT29 cells, while with the WEHI 164 line, recombinant mLT $\alpha/\beta$  was used to activate LT $\beta$ R. CBE11 was immobilized by capture with an anti-Fc mAb. In the HT29 assays, 80 U/ml of IFN- $\gamma$  were included, anti-FLAG was present with the hTRAIL and hFasL and anti-His-Tag was present with mFasL and mTRAIL to further crosslink these ligands. WEHI 164 cells were not sensitive to mFasL at concentrations up to 5  $\mu$ g/ml

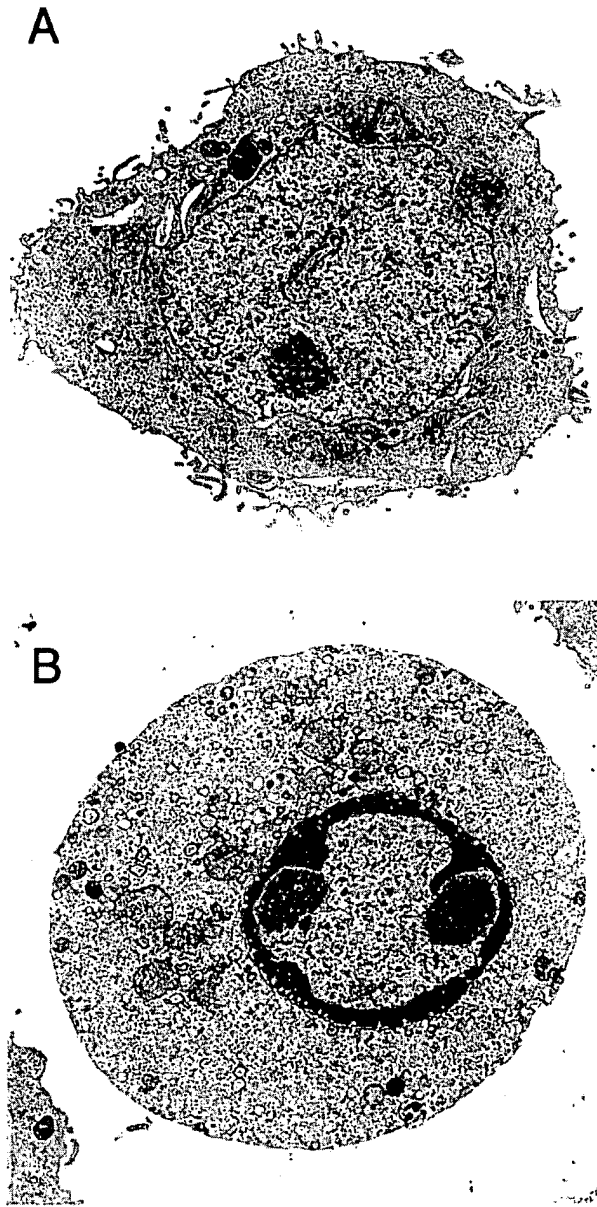
subdiploid DNA and this event was also completely blocked by caspase inhibition (Table 2). As previously reported and reproduced here, TNF did not induce DNA fragmentation in L929 cells undergoing necrosis. Fluorescence microscopy of DAPI stained nuclei was employed to visualize chromatin condensation. Chromatin condensation, i.e. smaller nuclei, was observed in anti-Fas or TNF treated HT29 cells both in the presence and absence of zVAD-fmk (Figure 3A,B) indicating that the death retained this feature of apoptosis. With anti-Fas, but not TNF, TRAIL or TWEAK treatment, there were fewer condensed nuclei in the presence of zVAD-fmk after 2 days of treatment suggesting that Fas induced death was slowed (data not shown, addressed further below). In contrast, zVAD-fmk effectively prevented chromatin condensation in WEHI 164 cells when triggered by either TNF-R (not shown) or LT $\beta$ R activation (Figure 3C). Thus chromatin condensation was caspase-independent in HT29 cells, but caspase-dependent in WEHI 164 cells.

In the presence of TNF alone, some U937 nuclei condense and break up into large fragments in a classic apoptotic pattern (Figure 3D). In the presence of TNF/zVAD-fmk, numerous U937 cells had smaller nuclei with altered morphology, i.e. condensed chromatin, yet chromatin breakup was not observed. This result differs from that reported for TNF induced L929 necrosis where cells die with an unaltered nuclear morphology.<sup>1</sup> TNF treated U937 cells undergo plasma membrane blebbing with a classical apoptotic appearance and inclusion of zVAD-fmk eliminated this blebbing. Thus TNF/zVAD-fmk induced U937 cell death was characterized by chromatin condensation, but without blebbing, extensive nuclear fragmentation or DNA cleavage to the nucleosomal level.

Translocation of phosphatidylserine was measured by annexin V binding in parallel with a determination of the loss of membrane permeability as revealed by 7-AAD uptake. Typically early apoptotic cells are defined as having externalized phosphatidylserine with retention of membrane integrity, although even these cells will eventually lose membrane integrity and become annexin V/7-AAD double

positive. Necrotic cells, generated by ethanol treatment, are also annexin V/7-AAD double positive (not shown). Examination of these five events revealed five different patterns of cell death (Figure 4). A small increase in the number of single positive annexin V cells was observed following IFN- $\gamma$  treatment of HT29 cells and double positive cells were absent (Figure 4). With combined TNF/IFN- $\gamma$  treatment, most annexin-V positive cells also stained with 7-AAD indicating that death was primarily necrotic by this criterion. While all three adherent lines, i.e. HT29, L929 and WEHI-164 cells exhibited similar necrotic patterns of annexin-V staining, the addition of zVAD-fmk led to three different results. Annexin V staining on HT29 cells was unaltered, the display of annexin-V on WEHI-164 cells was completely blocked and, as expected, annexin-V staining of L929 cells was dependent on zVAD-fmk treatment. The low level of early apoptotic cells in the TNF treated WEHI-164 cells was surprising and no increase in their numbers was found even at very early time points. While the stress of detachment of early apoptotic HT29 cells from the plastic could lead to 7AAD uptake and a loss of annexin-V single positive cells, L929 and WEHI cells are very easily released and less likely to undergo rupturing. In contrast to L929 death, TNF/zVAD-fmk treated U937 cells exhibited an increased population of annexin-V positive, 7-AAD negative cells when compared to TNF treatment alone (Figure 4). Staurosporine induces apoptosis and staurosporine-treated Jurkat and U937 cells exhibited the same levels of phosphatidylserine exposure as seen with anti-Fas treated Jurkat or TNF/zVAD-fmk treated U937 cells (data not shown). Therefore the amount of phosphatidylserine translocation in U937 cells was consistent with an apoptotic event. As expected, the annexin V single positive population did not appear in Jurkat cells treated with anti-Fas in the presence of zVAD-fmk. Predominantly annexin V/7-AAD double positive L929 cells were observed at all times which is consistent with a pure necrotic event as described.<sup>39</sup>

Confocal microscopy verified that annexin V was bound to the plasma membrane surface of both anti-Fas treated



**Figure 2** Electron microscopic analysis of the morphology of HT29 cells undergoing anti-Fas induced cell death. (A) Cells treated for 24 h with IFN- $\gamma$  alone (80 U/ml). (B) IFN- $\gamma$  plus anti-Fas mAb (100 ng/ml)

Jurkat cells and TNF/zVAD-fmk treated U937 cells (Figure 5A,B). Annexin V binding to TNF/zVAD-fmk treated L929 cells was localized to intracellular organelles in pattern basically identical to ethanol treated necrotic Jurkat, U937 or L929 cells (Figure 5C–F). Thus both FACS and confocal microscopic analyses confirm that phosphatidylserine externalization accompanies TNF/zVAD-fmk induced U937 death. In contrast to the study by Khwaja *et al*,<sup>6</sup> it was reported that the death of U937 cells in response to anti-Fas, TNF and etoposide treatment was blocked by caspase inhibitors.<sup>40,41</sup> We have repeated these experiments and confirmed that while zVAD-fmk effectively blocked the DNA fragmentation, the total percentage of annexin V positive

**Table 2** Effects of zVAD on DNA fragmentation following TNFR or Fas activation

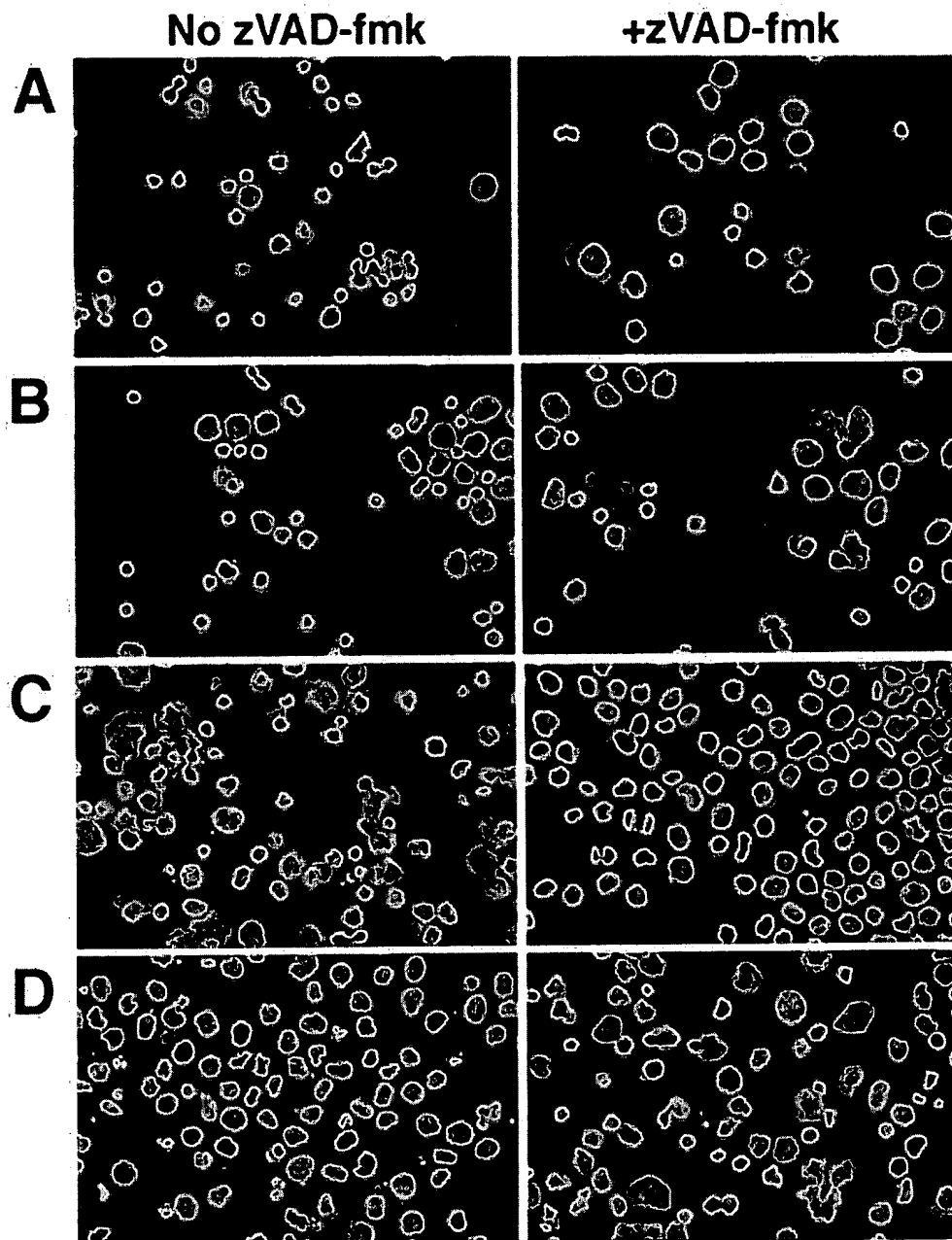
Cell	Receptor Activated <sup>a</sup>	Hours	Percentage of cells with subdiploid DNA +Agonist		
			Control	–zVAD	+zVAD
Jurkat	Fas	3	2	9	2
		5	7	84	6
		10	3	45	4
		15	7	27	6
U937	TNFR	3	0	0	0
		5	1	1	0
		10	1	2	1
		15	3	20	4
L929	TNFR	3	1	1	1
		5	2	2	2
		10	2	2	1
		15	3	5	7
WEHI 164	TNFR	5	1	3	4
		25	2	18	3
		48	1	51	1
		17	3	2	1
HT29	TNFR*	41	3	44	7
		17	3	3	1
	LT $\beta$ R	41	3	15	3

<sup>a</sup>Agonist for Fas was CH11 anti-Fas, TNF for TNFR and a pentameric form of an anti-LT $\beta$ R agonistic mAb. HT29 experiments were conducted in the presence of 80 U/ml of IFN- $\gamma$  and the HT29 control values represent cultures with IFN- $\gamma$

cells (including both 7AAD positive and negative) was unaltered following etoposide or anti-Fas treatment (data not shown). Likewise, when U937 cells were examined in a 4 day MTT growth assay, caspase inhibition did not spare the cells from the death or anti-proliferative effects of anti-Fas or etoposide treatment. Therefore, the death of U937 cells is caspase-independent when triggered by TNF-R, and Fas activation or etoposide.

### Collapse of mitochondrial membrane potential and involvement of reactive oxygen species (ROS) in these death events

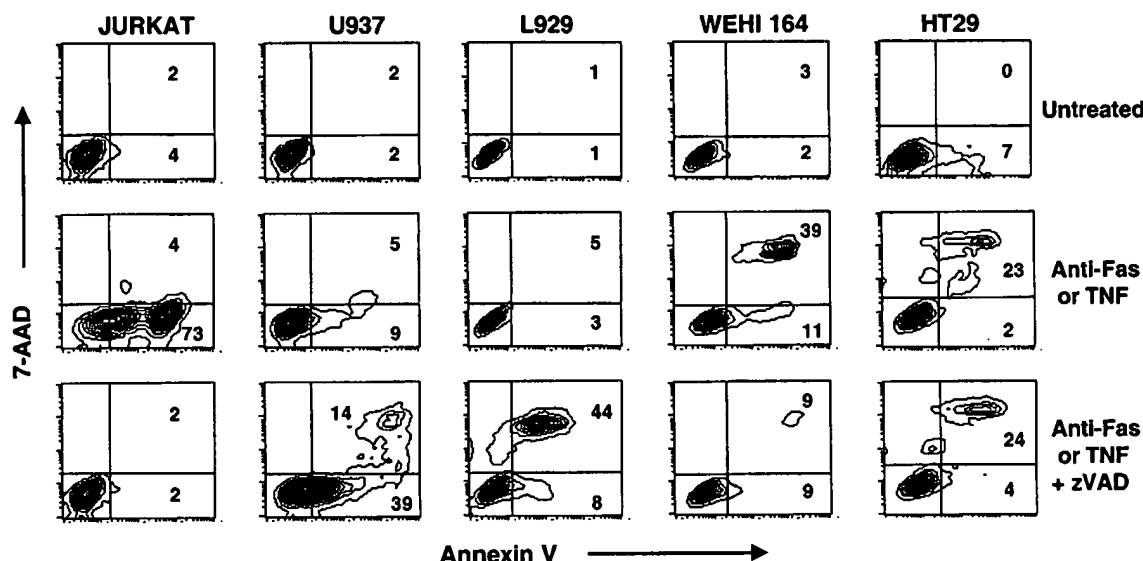
To compare these death events in more detail, we directly monitored as a function of time mitochondrial membrane potential, production of reactive oxygen species (ROS), annexin V binding and 7-AAD uptake as an indicator of the loss of membrane integrity. Decreased mitochondrial membrane potential is observed during apoptosis and is critical for the release of cytochrome c. The mitochondrial membrane potential can be quantitated by the loss of DiOC6 fluorescence, although this dye does monitor both plasma membrane and mitochondrial membrane potential.<sup>42,43</sup> Increased production of ROS has been implicated in other caspase-independent death events and can be detected in a FACS analysis by increased cellular staining with dihydroethidium.<sup>44</sup> As indicated from the study of annexin V binding, the five patterns of cell death were clearly different (Figure 6). As expected for the prototypical apoptotic event, the appearance of annexin V positive Jurkat cells paralleled the loss of mitochondrial potential and increased ROS production. Loss of Jurkat plasma membrane integrity lagged about 8 h behind the other changes. In contrast, annexin V single positive



**Figure 3** Analysis of the effects of zVAD-fmk (50  $\mu$ M) on chromatin condensation following TNF family receptor activation. HT29 cells were treated 48 h with anti-Fas mAb in (A) or TNF (20 ng/ml) (B). WEHI-164 cells were treated for 24 h with murine LT $\alpha$ / $\beta$  in (C) and U937 cells were exposed to TNF (10 ng/ml) for 24 h in (D). DAPI staining of the nuclei shows large normal nuclei or small condensed nuclei. Some mitotic figures are present in panel C (+zVAD-fmk). Magnification was 630  $\times$

staining of U937 cells treated with TNF/zVAD-fmk preceded the loss of mitochondrial membrane potential and membrane integrity and only very low levels of ROS were produced. TNF/zVAD-fmk treated L929 cells quickly produced high levels of ROS and lost membrane integrity in the complete absence of annexin V binding. This result is consistent with prior descriptions of a relatively pure necrotic event. TNF treated WEHI-164 cells did not make detectable levels of ROS yet lost both mitochondrial membrane potential and membrane integrity at the same time. Caspase inhibition blocked all changes in WEHI-164 except for some decrease in

mitochondrial membrane potential, but pre-treatment with zVAD-fmk completely blocked the membrane potential change (data not shown). Like L929 cells, HT29 cells produced ROS in parallel with decreased membrane potential and the loss of membrane integrity, but the entire process occurred more slowly. In contrast to L929 cells, this induction was not dependent on the inhibition of caspase activity. Activation of LT $\beta$ R in HT29 cells in the presence of IFN- $\gamma$  showed a similar pattern (not shown). From this analysis it is clear that the death of HT29 cells was not appreciably slowed by caspase inhibition. Similar results were obtained in an



**Figure 4** Annexin V binding defines different patterns of cell death in the Jurkat, U937, L929, WEHI 164 and HT29 systems. FACS analysis of FITC-annexin V binding to cells that were counterstained with 7-AAD to indicate loss of cell integrity. Cells were analyzed at 5 h post addition of anti-Fas (Jurkat), 5 h post addition of TNF for U937, L929 and WEHI 164 cells and at 27 h for HT29 cells. All agonists were present at 50 ng/ml and zVAD-fmk was at 50 nM. Numbers indicate the percentage of cells with the indicated gate. The cells in all three HT29 panels were treated with 80 U/ml of IFN- $\gamma$  and control cells without IFN- $\gamma$  had 2 and 1% of the cells in the double and single positive annexin V quadrants respectively

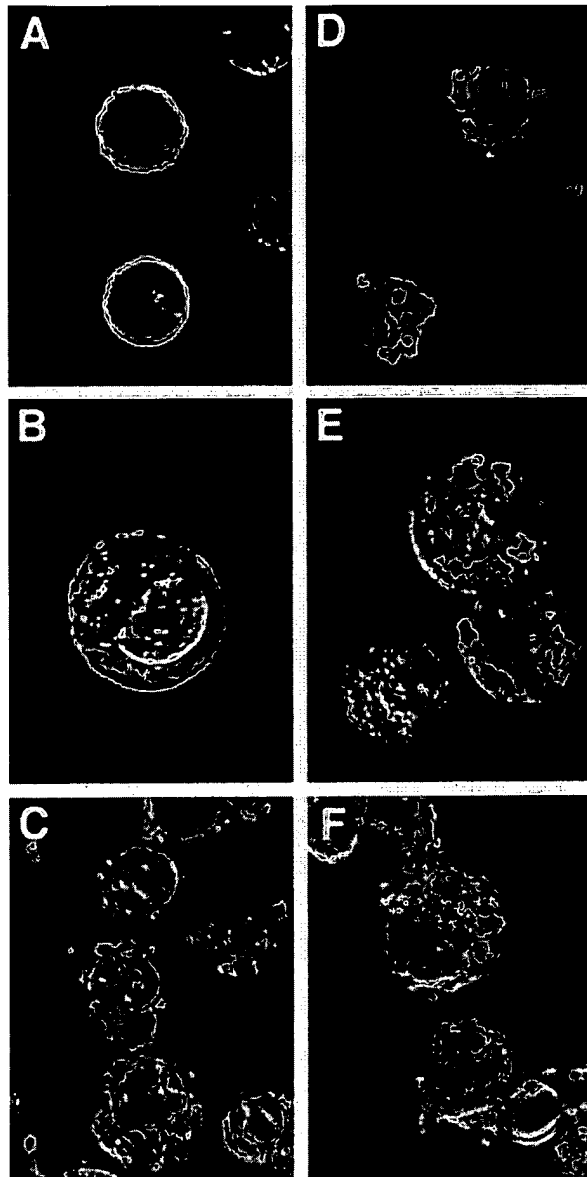
analysis of changes in the rate of acidification of the extracellular medium performed in real time using a Cytosensor<sup>®</sup> apparatus (data not shown). zVAD-fmk was found to delay TNF induced changes in proton fluxing by 5–6 h in HT29 cells yet the final outcome was not altered. Therefore, using two different techniques to monitor the kinetics of the death process, we find that the death in HT29 cells is only slightly slowed in the absence of caspase activity. These observations describe five phenotypically different forms of death in response to TNF receptor family activation. The TNF/IFN- $\gamma$ -HT29, TNF-WEHI-164 and TNF/zVAD-U937 deaths appear to be mixed forms with elements of both necrotic and apoptotic processes.

## Discussion

The presence of a death domain in a TNF type receptor is generally considered to be indicative of the potential to induce death. However, a number of TNFR family members lacking canonical death domains can trigger death in *in vitro* settings. Here we have studied TNF family triggered events in the HT29 colon epithelial tumor line in the absence of RNA or protein synthesis inhibitors and without overexpression of any transfected genes. These results document that this cell line dies in response to signaling through the TNF, TRAIL, Fas, TWEAK and LT $\beta$ R receptors in the absence of caspase activity. Second, the induced expression of a *bona fide* death signaling ligand like TNF does not account for death following LT $\beta$ R activation in both HT29 and WEHI 164 cells. Thus activation of LT $\beta$ R and most likely the TWEAK receptor can directly induce death in the absence of a death domain. Third, HT29 cell death displays characteristics of both apoptosis and necrosis and appears to be distinct from the other reference death events analyzed in this study.

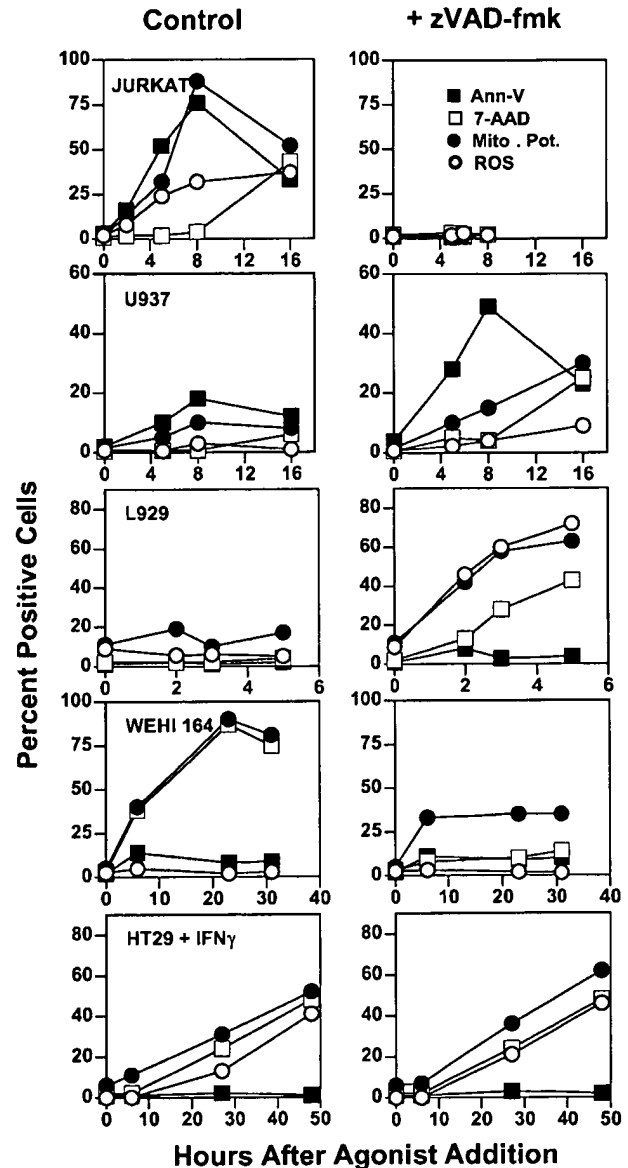
The ability of death domain-less TNF family receptors to trigger cell death has been enigmatic. The death accompanying activation of TNFR $\text{II}$ , LT $\beta$ R, CD27, CD30, CD40 and Taj falls into this group. In the case of TNFR $\text{II}$ , some *in vivo* evidence linked this receptor to death events.<sup>7,15,20,24,25,45–51</sup> It was reported that TNFR $\text{II}$  or TWEAK receptor activation in the rhabdomyosarcoma line KYM-1 induced membrane TNF expression which then triggered cell death in a classical manner via TNFR $\text{I}$  activation.<sup>7,8</sup> This observation was extended to the death of two cell lines overexpressing CD40 and partially to CD30 activation in the lymphoma derived Karpas-299 line. Therefore there was a potential explanation for this enigma that did not involve coupling of nondeath-domain receptors to the death machinery. It was noted that not all death following CD30 activation was accounted for by this mechanism.<sup>7</sup> In principle, the relatively slow nature of HT29 death would be consistent with a secondary mechanism, yet our previous observations with TWEAK indicated that HT29 death does not involve induction of TNF or FasL expression.<sup>20</sup> Similarly, neither TNF, FasL nor TRAIL inhibitors affected LIGHT induced HT29 death.<sup>21</sup> Fn14 has been shown to be a specific high affinity receptor for TWEAK and it lacks a death domain.<sup>52</sup> The lack of induction of a secondary ligand is consistent with the concept that there is a novel TNF family triggered death pathway in some cells. There are a couple of caveats to this conclusion. Induced-ligand receptor interactions and signaling could occur during protein synthesis and therefore would remain inaccessible to exogenously added blocking agents. It is quite difficult to completely exclude such internal signaling and likewise the existence of additional unknown ligands. Even if such secondary mechanisms are operative, HT29 death remains caspase-independent in-





**Figure 5** Annexin V binding to TNF/zVAD-fmk treated U937 and anti-Fas treated Jurkat cells reveals a similar apoptotic pattern. Cells were treated as defined in Figure 4, labeled with Alexa 488 annexinV (green) and propidium iodide (red) following 5 h exposure to the agonists and visualized by confocal microscopy: (A). anti-Fas treated Jurkat cells, (B). TNF/zVAD-fmk treated U937, (C). TNF/zVAD-fmk treated L929 and ethanol induced necrotic controls (D). Jurkat, (E). U937 and (F). L929 cells. A differential interference contrast image is overlaid in the background

dicating that there are other routes to cell death. Likewise, one can question the validity of using a chemical caspase inhibitor to define caspase independence; however, all the caspases known to couple TNF family receptors to downstream signal transduction machinery are zVAD sensitive. We cannot exclude the involvement of further unknown zVAD resistant caspases although it is likely that most caspases are now known and except for caspase-2, all are zVAD-fmk sensitive. Similar logic would apply to the use of cells transfected with crmA type protease inhibitors.



**Figure 6** Time course comparison of increased annexin-V binding, loss of mitochondrial membrane potential, ROS generation and increased cell permeability as determined by FACS analysis. From top to bottom, anti-Fas treated Jurkat, TNF treated U937, L929 and WEHI-164 and TNF/IFN- $\gamma$  treated HT29 cells in the absence and presence of 100 nM zVAD-fmk. Compared are single positive annexin V binding (filled square), percentage of cells with decreased DiOC<sub>6</sub> fluorescence as an indicator of decreased mitochondrial membrane potential (filled circle), percentage of cells with increased dihydroethidium fluorescence as a monitor of ROS production (open circle) and the 7-AAD fluorescence showing the loss of membrane integrity (open square)

The death of the exquisitely sensitive WEHI 164 fibroblastoid line following LT $\beta$ R activation was blocked by an anti-LT $\beta$ R mAb, but not by mTNFR1-Ig<sup>24</sup> and this work further excludes mFasL, mTRAIL and mTWEAK induction as a component of that activity. Based on this singular and rather complex system involving a heteromeric ligand, a death domain-less receptor appears to be able to couple to

caspase-mediated events. Direct coupling at the receptor level would be unlikely and the observation probably reflects engagement of downstream effector caspases. Similar caspase-dependent induction of apoptosis was triggered by the death-domainless TNFRII following supraoptimal antigen stimulation of the TCR and also accompanied overexpression of CD30 and LT $\beta$ R.<sup>51,53,54</sup> These observations substantiate the premise that the death-domainless receptors can initiate death events without proximal caspase involvement.

An emerging concept is that in some settings, TNFR family members induce an apoptotic death; however, the death becomes necrotic in the absence of functional caspases.<sup>1,4-6,57,58</sup> In HT29 cells, receptors in the TNF family trigger a death with features of both apoptosis and necrosis. The observations of chromatin condensation and margination, loss of microvilli, detachment and rounding are indicative of apoptotic events yet cytoplasmic shrinkage was not observed with HT29 cells. The observed organelle-free clear cytoplasmic blebs are probably more indicative of necrosis. Moreover, we did not observe an appreciable number of early phase apoptotic HT29 cells with annexin V binding as was the case with TNF/zVAD induced necrotic death of L929 cells. Since chromatin condensation occurred, this death differs from autophagic death which is not accompanied by changes in nuclear morphology.<sup>11</sup> We conclude that the death of HT29 cells triggered by receptors in the TNF family is a mixed form with features of both necrosis and apoptosis and fits most closely the apoptosis-like PCD described by Leist *et al.*<sup>9</sup> A previous study defined the HT29 death as caspase-dependent and this conclusion was reached from the quantitation of histone associated DNA fragments which correlate with nucleosome release following simultaneous exposure to rather high levels of TNF, IL-1 and IFN- $\gamma$ .<sup>35</sup> While caspase inhibition can slow TNFR and Fas triggered HT29 death and nucleosomal cleavage will be prevented, the cells still die. Many studies have relied on inhibition of DNA fragmentation as the readout of apoptosis, yet this linkage does not necessarily reflect the survival outcome. This work

shows that these events are clearly coupled in Jurkat and WEHI 164 cells, but in HT29 and U937 cells, using fragmentation as a surrogate marker for death leads to an erroneous conclusion. HT29 death may result from engagement of several death effector mechanisms. It is also possible that following TNFR, Fas or TRAIL-R activation, two or more pro-death mechanisms are engaged, one of which is caspase-mediated and a second pathway which is revealed in the presence of caspase inhibition.

To properly characterize the HT29 death event, we used anti-Fas treated Jurkat and TNF/zVAD-fmk treated L929 cells as reference points for prototypical apoptotic and necrotic events. TNF/zVAD induced U937 death was also investigated in some depth given its unusual nature along with the exquisitely sensitive WEHI-164 murine fibroblast line.<sup>6</sup> A summary of the properties of the various death events analyzed here is presented in Table 3. Analysis of loss of mitochondrial potential, chromatin cleavage and condensation, annexin V binding, ROS production and zVAD sensitivity revealed that the five death events were quite different. This heterogeneity of death types was surprising and it was difficult to find unifying features. Only the potentiation of TNF induced death by zVAD in NIH3T3, L929 and U937 cells suggested a common mechanism. The recent description of alternate Fas signaling depending on whether a caspase or FLIP serves as the primary adaptor may provide some molecular rationale for the potentiating effects of caspase inhibitors in some systems.<sup>57</sup> The U937 and L929 systems are also similar in outcome, yet differ substantially. The translocation of phosphatidylserine in dying U937 cells clearly demarcates this event from L929 necrosis. Curiously, we could not find many early apoptotic cells when death was induced in cells of nonhematopoietic lineage, which also happened to be adherent cell lines, yet such cells were readily observed in the suspended U937 and Jurkat systems. It is unclear if this is a generalizable observation given that only a few cells from each lineage were examined here. Likewise, membrane damage accompanying the removal of dying

**Table 3** Summary of the death events induced by TNF family signaling in five cell types

Cell	Receptor	Chromatin		A-V <sup>b</sup>	ROS	Type of death
	activated	Condensation	Fragmentation			
Jurkat	FAS	+	+++	+++	++	Apoptosis
	FAS+zVAD	-	-	-	-	No death
U937	TNFR	+/-	+	+/-	-	Apoptosis
	TNFR+zVAD	+	+/-	++	+	Mixed?
L929	TNFR	-	-	-	-	Little death
	TNFR+zVAD	-	-	-	+++	Necrosis
WEHI 164	TNFR	+	++	+	-	Apoptosis
	TNFR+zVAD	-	-	-	-	No death
	LT $\beta$ R	+	++	+	-	Apoptosis
	LT $\beta$ R+zVAD	-	-	-	-	No death
HT29	TNF <sup>a</sup>	+	++	+/-	++	Mixed
	TNF+zVAD	+	-	+/-	++	Mixed
	LT $\beta$ R	+	++	+/-	++	Mixed
	LT $\beta$ R+zVAD	+	-	+/-	++	Mixed

<sup>a</sup>Similar data for activation of each of the following receptors, TNFR, Fas, TWEAK-R, TRAIL-R and LT $\beta$ R. <sup>b</sup>Annexin V positive, 7AAD negative early apoptotic cells

cells from plastic may mask the existence of early apoptotic cells. Our attempts to prestain or remove the cells with trypsin without EDTA-induced trauma did not result in enhanced detection of annexin V single positive adherent cells (data not shown). Likewise in HT29 and U937 cells, we observed chromatin condensation without DNA fragmentation indicating that chromatin condensation is a separate event independent of caspase activation, a conclusion reached by others.<sup>58</sup> This observation may also be consistent with DNA cleavage down to 50 kb fragments being critical for condensation since this event is mediated by AIF and/or Acinus which can be activated independently of caspase activity.<sup>58,59</sup> This level of chromatin condensation may be part of an apoptotic program or it may be linked to cell cycle events and mitotic catastrophe. In contrast, chromatin fragmentation and condensation appeared to be coupled in Jurkat and WEHI-164 cells. Therefore using the Jurkat and L929 systems as examples of pure apoptosis and necrosis, the TNF-HT29, TNF/zVAD-U937 and TNF-WEHI 164 deaths were mixed with varying degrees of apoptotic and necrotic features. These results suggest that TNF receptor family members can engage various components of the death machinery selectively, leading to the various death phenotypes described here.

Reactive nitrogen species and ROS generation probably play central roles in many death processes and the TNF/zVAD treated L929 cases may be a good example.<sup>1</sup> In HT29 cells, ROS production was significant and paralleled the loss of membrane potential. Attempts to protect HT29 cells from death with antioxidants have not been successful in our hands. Others have noted that agents such as butylated hydroxyanisole only poorly protect cells from late phase ROS components and, moreover, it is unclear whether these ROS indicator dyes such as dihydrorhodamine or dihydroethidium report faithfully on all ROS forms.<sup>60</sup> A novel fullerene based antioxidant was noted to block HT29 death following LT $\beta$ R activation by the ligand LIGHT which is supportive of an ROS based component.<sup>61</sup> Involvement of reactive nitrogen intermediates and ceramide have not been implicated and we also have not been able to demonstrate any involvement of these mediators.<sup>35,62</sup> ROS production was observed in TNF/zVAD treated NIH3T3 cells, yet was not reported for U937 cells.<sup>6</sup> In our U937 experiments we did not detect levels of ROS production comparable to the L929 or HT29 systems. While U937 ROS was slightly augmented by caspase inhibition, this increase only occurred after cells had begun to lose membrane integrity. When coupled with the differences in phosphatidylserine translocation, it is possible that the U937 death mechanism is fundamentally different from that engaged in HT29, L929 and NIH3T3 cells. Given the commonality of ROS induction, it would be attractive to group mechanistically the HT29, L929 and NIH 3T3 events. This step may be premature. First, the apparently mixed apoptotic/necrotic HT29 death is not dramatically potentiated by caspase inhibition. Secondly, the HT29 death is relatively slow and the requirement for IFN- $\gamma$  is unique. Lastly, the ability of zVAD-fmk to potentiate death is limited to the TNF receptor in L929 and U937 cells, e.g. TRAIL-R or LT $\beta$ R activation was not potentiated in U937 cells. In contrast, this death pathway

in HT29 cells can be generically triggered by probably all TNF family receptors present on these cells.

An analysis of the signal transduction pathways involved in HT29 death was not within the scope of this work and this area remains undefined. Earlier RNA based work had noted that IFN- $\gamma$  upregulated caspase expression in HT29 cells, yet here we have shown that caspases at most simply accelerate the HT29 death.<sup>19</sup> Moreover, in this study and in another report, the increase in caspase activity was very small compared to that occurring in both U937 and Jurkat cells.<sup>35</sup> Theoretically, one needs to invoke the involvement of a p53 independent pathway that can be commonly accessed by many TNF family receptors. Potential candidates include linkage to bcl-2 family members, phospholipases A2, PML induction or the stress activated protein kinases and these events are then coupled in some manner to AIF release and ROS generation.<sup>63,64</sup> cJun N terminal kinase (JNK), ERK and p38 kinase are activated by many TNF family receptors both with and without death domains often independently of caspase activation.<sup>57,65</sup> (including LT $\beta$ R, M Lukashev and M Jarpe, unpublished observations). TRAF adaptors have been suggested to link LT $\beta$ R to HT29 death and are potentially involved in linking this receptor to NF $\kappa$ B $\gamma$  inducing kinase.<sup>66,67</sup> Mitochondrial AIF release is an attractive candidate to account for these observations and the receptor may be coupled to the mitochondria via effects on bcl-2 family members or via kinase activation. Interestingly, both the morphology and the caspase-independence of the HT29 death event resemble the death of tumor cells following depletion of Hsp70.<sup>68</sup> Moreover, Hsp70 overexpression has been shown to block AIF induced apoptosis suggesting some parallels in these observations.<sup>69</sup>

We have shown here that TNFR family members can trigger HT29 death via a caspase-independent pathway regardless of the presence or absence of a death domain in the receptor. This death displays aspects of both apoptotic and necrosis. Whether these events are physiologically relevant remains unknown; however, the human adenocarcinoma HT29 line is a pluripotent fetal-like epithelial cell representative of many primary colon carcinomas.<sup>28</sup> The death of HT29 epithelial cells when triggered especially by LT $\beta$ R is relatively slow and, while more difficult to study, it may be revealing previously unappreciated behavior. An understanding of these slower death events in epithelial cells may prove useful for the development of novel anti-cancer approaches. Certainly activation of TNF family receptors lacking death-domains should be evaluated in oncology settings. A better comprehension of how transformation, differentiation, IFN- $\gamma$  signaling and tumor suppressor gene status influences the manner in which a cell interprets signals from the TNF family receptors may help define tumors susceptible to this type of treatment.

## Materials and Methods

### Cells and reagents

The HT29-14 variant of ATCC derived HT29 cells has been described<sup>15</sup> and the Jurkat, U937 and L929 cells were obtained from

the ATCC and grown in RPMI 1640 medium with 10% fetal bovine serum (Hyclone, Logan, UT, USA) and glutamine. The origin and growth of the WEHI 164 line has been described.<sup>70</sup> zVAD-fmk was obtained from Bachem (Torrance, CA, USA). The following ligands and antibodies were purchased: anti-human Fas mAb CH11 (Kamaya Biomedical Co, Seattle, WA, USA), neutralizing anti-human Fas-L (NOK-1) and anti-murine Fas-L (MLF3) (BD Pharmingen, San Diego, CA, USA), anti-murine TNF neutralizing mAb, MP6-XT3 (Gibco BRL, Gaithersburg, MD, USA), murine TRAIL-flag (Biomol Research Lab, Plymouth Meeting, PA, USA), murine His tagged-Fas ligand and anti-His Tag (R&D Systems, Minneapolis, MN, USA) human FLAG-Fas ligand (Alexis, San Diego, CA, USA), anti-Flag M2 (Sigma, St. Louis, MO, USA) and FITC labeled annexin V (Pharmingen, San Diego, CA, USA). Human FLAG-TRAIL and human TRAIL-R2-Ig were gifts from Jurg Tschopp. MP6-XT3 had been shown to effectively block murine TNF.<sup>24</sup> The hamster anti-TWEAK antibody ABD3 was developed at Biogen and effectively blocks both human and murine TWEAK-induced HT29 cell death.<sup>71</sup> The human IFN- $\gamma$ , anti-human TNF mAb 104c, murine and human LT $\beta$ R-Ig fusion proteins, the human and mouse LT $\alpha$ / $\beta$  ligands and the agonist anti-human LT $\beta$ R mAb (CBE11) have been described previously.<sup>15,24,72</sup>

## Microscopy

Electron microscopy was performed on samples that included all the cells, i.e. both the dead cells released from the plastic surface and the remaining adherent cells obtained by trypsinization. Microscopy was performed on thin sections using conventional paraformaldehyde fixation and osmium tetroxide staining. For fluorescence microscopy, cells were fixed in 2% paraformaldehyde, cytospun onto slides, fixed in 95% ethanol and stained with 0.5  $\mu$ g/ml DAPI. In the confocal microscopy experiments, cells were stained with Alexa-488 annexin V (Molecular Probes, Eugene, OR, USA) as described by the manufacturer and 5  $\mu$ g/ml propidium iodide.

## Cell growth assays

The growth assays involved plating 3000–5000 cells per 96-well depending on the cell type and viable mitochondria were quantitated with a MTT readout after 3–4 days of growth. These assays have been previously described in detail.<sup>20</sup> Cells were mixed with 100  $\mu$ M zVAD-fmk for 10 min prior to dilution into the assay yielding a final concentration of 50  $\mu$ M during the growth phase. zVAD-fmk was dissolved at a stock concentration of 20–100 mM in DMSO and matching solvent concentrations were run as controls in all experiments. The input cell numbers for the long-term growth assays were 5000/well for HT29, Jurkat and U937 cells and 4000/well for WEHI 164. For anti-LT $\beta$ R agonist antibody killing of HT29 cells, serial dilutions of the antibody were captured onto goat anti-mIgGFC-coated 96-well plates (10  $\mu$ g/ml). In blocking experiments, the following inhibitor concentrations were used: anti-hTNF 104c, MP6-XT3, BBF6 anti-mLT $\beta$ , ABD3 anti-h/mTWEAK and anti-hFasL NOK-1 at 5  $\mu$ g/ml, anti-mTNF and MLF3 anti-mFasL at 3  $\mu$ g/ml and hTRAIL-R2-Ig, mTRAIL-R2-Ig and hLTBR-Ig at 2  $\mu$ g/ml.

## FACS analyses

L929 cells were removed by EDTA treatment and WEHI-164 and HT29 cells were gently subjected to a mild trypsinization for removal. All floaters were included in the analysis. Cells were resuspended at a concentration of  $10^6$ /ml in complete tissue culture media in the presence or absence of 250 ng/ml anti-Fas mAb CH-11, 100 ng/ml or TNF,  $\pm$  50  $\mu$ M zVAD-fmk. Following the 5 h treatment, L929 and WEHI

164 cells were removed again by EDTA and washed once. Jurkat and U937 cells were harvested directly. For determination of annexin V binding and membrane permeability, the cells were resuspended in 50  $\mu$ l of cold RPMI with 10% FBS, 0.05% azide and 50  $\mu$ l of 2  $\mu$ g/ml FITC labeled annexin V in media was added for 30 min on ice. Cells were then resuspended in 100  $\mu$ l of 10  $\mu$ g/ml 7-amino actinomycin D (7-AAD) in media with azide for another 5 min, washed in media with azide and analyzed in Ca<sup>2+</sup>/Mg<sup>2+</sup> containing PBS with 5% FBS and 0.05% azide. Cells were placed in 10% ethanol for 3–5 h at 37°C to provide a positive control for necrosis.<sup>73</sup> Aliquots of cells at each timepoint were resuspended into hypotonic propidium iodide, left overnight at 4°C and analyzed for DNA fragmentation by conventional FACS methods. For determination of superoxide production and mitochondrial potential changes following anti-Fas or TNF treatment,  $2 \times 10^5$  cells were treated with 250 nM dihydroethidium or 25 nM DiOC<sub>6</sub> (Molecular Probes, Eugene, OR, USA) for 40 min at 37°C. The cells were washed once and analyzed on a FACS Calibur Instrument using the FL1 and FL3 channels for DiOC<sub>6</sub> and dihydroethidium, respectively. The data are presented as percent positive cells which was actually either decreased DiOC<sub>6</sub> or increased dihydroethidium staining and only very small cells and debris in the scatter profile were gated out.

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